Further Characterization of Platelet-Type von Willebrand’s Disease in Japan

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We studied four patients who showed aggregation of platelets in platelet-rich plasma at lower concentrations of ristocetin than those required for normal platelet-rich plasma and who demonstrated an increased capacity of the platelets to bind normal von Willebrand factor. The four patients were from two Japanese families. Platelets from one family aggregated spontaneously in vitro, and platelets from both families aggregated upon the addition of normal plasma and cryoprecipitate, in the absence of ristocetin or other agonists. Analysis of the multimeric composition of von Willebrand factor by sodium dodecyl sulfate-agarose gel electrophoresis revealed a decrease in large multimers or a decrease in both large and intermediate multimers in plasma, but normal multimers in platelets. 1-Deamino-[8-D-arginine]-vasopressin caused an immediate appearance of larger multimers in plasma, followed by the rapid disappearance of these multimers from circulating plasma. Analysis of platelet membrane glycoproteins from the patients showed that there were two distinct bands in the glycoprotein IIb region: one migrated in a slower region and the other in a faster region than normal glycoprotein IIb. We suggest that the platelet receptor abnormality in these patients is related to this abnormality of glycoprotein IIb.

VON WILLEBRAND’S DISEASE (vWD) is a heterogeneous disorder.1 Except for the homozygous severe form (type III), the more common and milder forms are classified as type I or type II, based on the multimeric structure of von Willebrand factor (vWF).1-4 In type I vWD, the multimeric composition of vWF is normal in plasma and platelets, but plasma vWF is quantitatively decreased. Ristocetin-induced platelet aggregation (RIPA) in platelet-rich plasma (PRP) is decreased due to reduced concentrations of all multimeric forms of vWF. Type II vWD is characterized by an absence of the large vWF multimers from plasma and can be further subdivided into type IIA, type IIB, and type IIC. In type IIA vWD, only the smaller vWF multimers are present in plasma and platelets. RIPA in PRP is markedly decreased or absent because the binding of this vWF to platelets is minimal or absent. In type IIB vWD, described by Ruggeri et al.,2,3 the large multimers are missing from plasma, as in type IIA. However, type IIB differs from type IIA in that intermediate forms are present in plasma, and multimeric composition of vWF in platelets is normal. Both RIPA in PRP and binding of this vWF to normal platelets occur at lower concentrations of ristocetin than in normal subjects. Type IIC is a recently described form of vWD characterized by lack of the large multimers of vWF in both plasma and platelets and by an aberrant triplet structure of each vWF multimer.5

In 1980 we also described two families in whose members smaller quantities of ristocetin induced aggregation of platelets in PRP than are required for normal PRP, and plasma findings were similar to type IIB vWD in regard to ristocetin cofactor activity (VIIIIR:RCo) and to the results of crossed-immunoelectrophoresis.6-8 In contrast to type IIB, a platelet abnormality was suggested in these patients as the pathogenesis of heightened interaction between platelets and vWF. The platelets from these patients bound more vWF in the presence of ristocetin than did normal platelets.7 Recently, Weiss et al.9 and Miller and Castella10 reported similar families.

In this article, additional findings in our patients are reported in order to clarify the pathophysiology of this disorder. Platelets aggregated upon the addition of normal plasma, and partial normalization of multimeric composition of vWF in plasma was observed following administration of 1-deamino-[8-D-arginine]-vasopressin (DDAVP). Furthermore, platelet membrane glycoprotein I was abnormal in these patients.

MATERIALS AND METHODS

Blood was collected into siliconized tubes containing 1/10 vol of 0.129 mol/L trisodium citrate. Platelet-rich plasma was prepared from the citrated whole blood by centrifugation at 175 g for ten...
minutes at room temperature, and platelet-poor plasma (PPP) was obtained by centrifugation at 2,000 g for 20 minutes at 4 °C.

Cryoprecipitate was prepared from citrated normal plasma. The plasma was frozen and stored at −70 °C and thawed at 4 °C overnight. The precipitate was centrifuged at 2,000 g for 20 minutes at 4 °C and the supernatant was removed. The pellet (cryoprecipitate) was dissolved in 0.01 mol/L Tris-HCl, 0.15 mol/L NaCl, pH 7.4, containing 10.6 mmol/L trisodium citrate, to one eighth of the original volume of plasma. A lyophilized cryoprecipitate (Cryonichiyaku) was obtained from Nihon Seiyaku Co, Tokyo.

For studies of the multimeric structure of vWF in platelets, nine parts of blood were drawn into one part of 77 mmol/L Na2 EDTA. PRP was prepared as described above. The platelets in PRP were washed four times with Krebs-Ringer buffer containing 9 mmol/L Na2 EDTA. The final platelet suspensions were lyzed by rapid freezing and thawing five times and were centrifuged at 3,000 g for 20 minutes. The multimeric structure of vWF in platelets was studied in the supernatant.

Bleeding time was estimated by Duke's method. Platelet counting was performed by an electronic counter (Thrombocounter-C, Coulter Electronics Ltd, Hialeah, Fla) and/or phase-contrast microscopy. Platelet volume in PRP was estimated by Sysmex Microcellcounter CC-108 and PDA 400 (Toa Medical Electronics Co, Kobe, Japan). Platelet aggregation by adenosine diphosphate (ADP; Sigma Chemical Co, St Louis, Mo), epinephrine (Daichii Seiyaku, Tokyo), collagen (Hormon-Chemie, München, West Germany), and ristocetin (H. Lundbeck Co, Copenhagen) was performed in PRP by the turbidimetric method of Born using a NKK aggregometer. The delay was minimal in family B as compared with family A. The multimeric structure of vWF in platelets was studied (Table 1).

RESULTS
Platelet Counts and Platelet Volume
Platelet counts were always normal in patients 1 and 2 (family A). Patients 3 and 4 from family B showed persistent thrombocytopenia during six years of observation. Platelet volume was increased in three patients studied (Table 1).
Spontaneous Platelet Aggregation and Platelet Aggregation by Human vWF

To investigate spontaneous platelet aggregation, PRP from the patients or normal subjects was stirred in the aggregometer cuvette without addition of any aggregating agents. As shown in Fig 1, patients 3 and 4 exhibited spontaneous platelet aggregation. The platelet count in PRP was only \(225 \times 10^9/L\) in patient 1 and was completely inhibited by aspirin (6 mmol/L) and was completely inhibited by EDTA (2 to 6 mmol/L). In the presence of 2 mmol/L EDTA, platelet aggregation induced by normal plasma was still minimally observed in patient 3, but it was completely abolished by 6 mmol/L EDTA. Heparin (1 U/mL) slightly inhibited this aggregation in patients 3 and 4, but had no effect on the magnitude of aggregation in patient 1 (Fig 3). Cryoprecipitate prepared from normal subjects and a lyophilized cryoprecipitate (Cryo-Nichiyaku) also induced platelet aggregation of patients' PRP in a dose-dependent manner (Fig 4). However, cryosupernatant failed to induce the aggregation.

Platelet Membrane Glycoproteins

Glycoprotein profiles of platelet membranes from the patients are shown in Figs 5 and 6. The glycoprotein nomenclature used was that proposed by Phillips and Agin\(^{24}\) and calculated molecular weights of GPIa, GPIb, GPIIb, GPIII, and GPIV were similar to those reported by these authors\(^{24}\): GPIa 167,000, GPIb 143,000, GPIIb 132,000, GPIII 114,000, and GPIV 97,000 in reduced samples. In all four patients, GPI was abnormal, and two distinct glycopeptides were demonstrated in the gpl region; one migrated slower and the other migrated faster than normal GPIb. These abnormal bands were called GPI\(^{1}\) and GPI\(^{1}\). No obvious band was observed in the region corresponding to normal GPIb. The calculated molecular weights of GPI\(^{1}\) and GPI\(^{1}\) were 150,000 and 140,000, respectively. These abnormal bands were equally obtained in either reduced or nonreduced platelet samples (Fig 7). These experiments were performed four times on different days, using fresh samples collected on each day, with the same results.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Family</th>
<th>Sex</th>
<th>Bleeding Time (min)</th>
<th>Platelet Count ((x 10^9/L))</th>
<th>Platelet Volume (fl) Mean</th>
<th>Platelet Volume (fl) Median</th>
<th>Platelet Volume (fl) Mode</th>
<th>Plasma FVIII/vWF (U/dL) VIII:C</th>
<th>VIIIIR:Ag</th>
<th>VIIIIR:RC0</th>
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<tr>
<td>1 A</td>
<td>M</td>
<td>F</td>
<td>&gt;20.0</td>
<td>225-326</td>
<td>10.4</td>
<td>9.6</td>
<td>6.9</td>
<td>43-62</td>
<td>48-110</td>
<td>18-35</td>
</tr>
<tr>
<td>2 A</td>
<td>M</td>
<td>F</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>32-42</td>
<td>51-55</td>
<td>17-41</td>
</tr>
<tr>
<td>3 B</td>
<td>M</td>
<td>F</td>
<td>&gt;20.0</td>
<td>47-91</td>
<td>11.8</td>
<td>12.3</td>
<td>9.6</td>
<td>54-110</td>
<td>79-135</td>
<td>36-57</td>
</tr>
<tr>
<td>4 B</td>
<td>M</td>
<td>M</td>
<td>4.5</td>
<td>74-110</td>
<td>10.8</td>
<td>10.2</td>
<td>8.1</td>
<td>69-82</td>
<td>72-73</td>
<td>40-45</td>
</tr>
</tbody>
</table>

Normal subjects

Mean: 272
Range: <5.0
No.: 212

ND: not determined.

*Platelet count and platelet volume are expressed as arithmetic means with 95% confidence limits, and factor VIII/von Willebrand factor (FVIII/vWF) related activities as geometric means with 95% confidence limits.

Fig 1. Aggregometer tracings of spontaneous platelet aggregation. Platelet-rich plasma from the patients was stirred in an aggregometer cuvette without any aggregating agents. The platelet count in platelet-rich plasma was 120 \(\times 10^9/L\) in patients 3 and 4, and was 350 \(\times 10^9/L\) in patient 1. Baseline (0%) and 100% aggregation were established by light transmission through platelet-rich plasma and platelet-poor plasma, respectively.

Fig 2. The aggregation of patient platelets by normal plasma. Normal plasma was added to an equal volume of platelet-rich plasma from the patients or a normal subject, after which the cuvette was placed in the aggregometer and baseline was established at 0% transmittance (100% transmittance being set with a mixture of an equal volume of normal plasma and platelet-poor plasma from the subjects tested) and aggregation was monitored.
Fig 3. Effects of prostacyclin (PGI₂), EDTA, aspirin, and heparin on platelet aggregation induced by normal plasma. Platelet-rich plasma (0.1 mL) from the patients (patients 3 and 4) was incubated with PGI₂ (10 nmol/L final concentration), EDTA (2 mmol/L), aspirin (6 mmol/L), heparin (1 U/mL) or normal saline (20 μL each) for three minutes at 37°C. Just after 0.1 mL of normal plasma was added, the cuvette was placed in the aggregometer and aggregation was monitored.

Examination of platelets from 20 normal subjects and an unaffected family member failed to demonstrate similar changes (Fig 7). Autoradiography of the gels, in which normal and patient platelets had been treated with neuraminidase, galactose oxidase, and sodium [³H]borohydride, also exhibited the two clearly separated bands in the GPI region (Fig 8). No apparent abnormalities were found in GPIIb, GPIII, and GPIV.

Multimeric Structure of Plasma and Platelet vWF

The multimeric structure of vWF was analyzed by SDS-agarose electrophoresis (Figs 9 and 10). In patients 1 and 2 from family A, the large and intermediate multimers were markedly decreased in plasma. Patients 3 and 4 from family B showed decreased large vWF multimers in plasma, but a considerable amount of intermediate forms was present. All patients showed a relative increase in small multimers. Difference of the multimeric composition in plasma vWF between family A and family B was apparent in both gels.

Fig 4. Aggregometer tracings of platelet aggregation induced by cryoprecipitate. Cryoprecipitate (Cryo-Nichiyaku) diluted with normal saline was added to an equal volume of platelet-rich plasma (227 x 10⁶ platelets/L) from patient 3, after which the cuvette was placed in the aggregometer, baseline was established, and aggregation was monitored. The amount of cryoprecipitate in the mixture is indicated in terms of units of VIIIIR:Ag and is given at the end of each trace. Under these conditions, no platelet aggregation was observed when normal saline was added to her platelet-rich plasma in place of cryoprecipitate.

Fig 5. Direct photography of platelet membrane glycoproteins from patients 1 and 2 and a normal subject (N). Reduced samples were electrophoresed in a 5.5% polyacrylamide gel in the presence of sodium dodecyl sulfate. The gel was stained with periodic acid-Schiff reagent and photographed. Abnormal glycoproteins observed in the patients in the glycoprotein I region were indicated as GPI' and GPI". TSP denotes thrombospondin.

Fig 6. Glycoprotein profiles of platelet membranes from patients 1 through 4 (A through D) and a normal subject (E). Reduced samples were electrophoresed in a 5.5% polyacrylamide gel in the presence of sodium dodecyl sulfate. The gel was stained with periodic acid-Schiff reagent and scanned in a densitometer at 550 nm. Abnormal glycoproteins observed in the patients in the glycoprotein I region were indicated as I' and I".
Fig 7. Glycoprotein profiles of platelet membranes in the reduced state (left) and nonreduced state (right) from patient 3 (A), her mother (unaffected) (B), and a normal subject (C). After electrophoresis in a 5.5% polyacrylamide gel, the gels were stained for carbohydrate with periodic acid-Schiff reagent and scanned in a densitometer at 550 nm. Abnormal glycoproteins observed in the patient in the glycoprotein I region were indicated as I' and I''.

which contained two different concentrations of agarose (2% and 1.3%). The multimeric composition of patient platelet vWF was indistinguishable from that in normal platelets (Fig 9). In Fig 9, the fastest migrating band of the triplet in each multimer was increased in quantity in plasma (but not in platelets) from patient 1, as seen in type IIA patients. However, it is unknown whether fresh plasma also shows a similar finding, because these studies were performed using once-frozen samples.

Changes in Multimeric Composition of Plasma vWF After DDAVP Infusion

DDAVP infusion into patients 1 and 3 was followed by about a two-fold increase in vWF-related activities and a shortening of the bleeding time. The bleeding time was greater than 20 minutes before DDAVP infusion in both patients, and it became 5.5 and 8.0 minutes in patients 1 and 3, respectively, five minutes after the end of infusion. Bleeding time returned to basal values within six hours after the infusion.

Fig 9. Multimeric composition of von Willebrand factor in plasma and platelets. Sodium dodecyl sulfate-agarose electrophoresis was performed with plasma and platelet lysates from a normal subject (N) or patients 1, 3, and 4. The agarose concentration in the running gel was 2.0%. The point of application is at the top, and the anode is at the bottom of the gel. The autoradiograph of von Willebrand factor in platelets from patient 3 was not intense, because the sample applied was too diluted (see also Fig 12).

Fig 10. Multimeric composition of plasma von Willebrand factor from a normal subject (N) and patients 1 through 4 studied at an agarose concentration of 1.3% in the running gel. The anode is at the bottom of the gel.
When analyzed by SDS-agarose electrophoresis, an increase in the amount of the larger multimers was demonstrated in plasma after DDAVP infusion (Figs 11 and 12). In plasma obtained five minutes after the infusion, the multimeric composition of vWF was partially normalized, although there was still a relative diminution in large multimers. The disappearance of newly released larger multimers from the circulation was very rapid, with some clearing of the large multimers already evident at one hour (Figs 11 and 12). The plasma levels of VIIIIR:Ag and VIIIIR:RCo reached a maximum at one or two hours, when larger multimers had already decreased. It was also true that after six hours, the multimeric composition was still different from baseline profile. Autoradiograph pattern of plasma vWF was also examined 24 hours after the infusion in patient 3, demonstrating that the multimer size distribution did not back to baseline (not shown). We did not obtain blood samples thereafter.

DISCUSSION

There are two distinct conditions in which RIPA in PRP and the binding of plasma vWF to platelets occur at lower concentrations of ristocetin than are required for normal subjects. One is type IIB vWD, in which plasma and platelet vWF is qualitatively (or functionally) abnormal and possesses an increased affinity for platelet binding sites (abnormal vWF protein). Another is the disorder described in this article (abnormal platelets), which is characterized by binding of normal vWF to patient platelets at lower concentrations of ristocetin than are required with normal platelets. Recently, similar patients have been reported by other investigators (Table 2) and have been designated as pseudo-vWD or platelet-type vWD.

Two of four patients studied here showed thrombocytopenia, but the other two patients did not. The patients described by Weiss et al showed intermittent thrombocytopenia, and another family described by Miller and Castella represented a borderline thrombocytopenic state. The platelets in PRP from our patients were aggregated by normal plasma or by normal cryoprecipitate (Figs 2 and 4), confirming the previous finding by Weiss et al. In addition, cryosupernatant failed to induce the aggregation, suggesting that these aggregations were vWF-dependent. Miller et al reported that although all their patients showed aggregation in response to cryoprecipitate or highly purified vWF, only one of four patients showed platelet aggregation when an approximately equal volume of normal plasma was added to their PRP. These differences among patients suggest that the threshold concentration of vWF in normal plasma that induces platelet aggregation would be variable among the patients. Platelet aggregation induced by vWF was completely inhibited by EDTA and partially inhibited by aspirin (Fig 3), demonstrating that the aggregation is dependent on divalent cations and is accompanied by the release reaction. These in vitro findings also imply that there might be a risk of in vivo platelet aggregation following therapeutic infusion of normal plasma or cryoprecipitate in these patients.

In agreement with previous reports, there was an absence of large vWF multimers in plasma from our
Table 2. Reported Cases of von Willebrand’s Disease With Abnormal Platelets

<table>
<thead>
<tr>
<th>Source</th>
<th>Weiss et al (one family)</th>
<th>Miller et al (one family)</th>
<th>Takahashi et al (Present Study)</th>
<th>Granlinc et al (two families)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Family A</td>
<td>Family B</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>Intermittent</td>
<td>Normal</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Platelet volume</td>
<td>Normal</td>
<td>ND</td>
<td>Increased</td>
<td>Increased</td>
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<tr>
<td>Spontaneous platelet aggregation</td>
<td></td>
<td></td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>RIPA</td>
<td>Increased*</td>
<td>Increased*</td>
<td>Increased*</td>
<td>Increased*</td>
</tr>
<tr>
<td>Platelet aggregation by normal plasma or normal vWF</td>
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<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Binding of normal vWF to patients’ platelets</td>
<td>Increased†</td>
<td>Increased†</td>
<td>Increased†</td>
<td>Increased†</td>
</tr>
<tr>
<td>Binding of patients’ vWF to normal platelets</td>
<td>Decreased</td>
<td>Normal</td>
<td>Decreased</td>
<td>Normal or decreased</td>
</tr>
<tr>
<td>Multimeric composition of vWF in plasma</td>
<td>Absence of large multimers</td>
<td>Absence of large multimers</td>
<td>Decrease in large and intermediate multimers</td>
<td>Absence of large multimers</td>
</tr>
<tr>
<td>Multimeric composition of vWF in platelets</td>
<td>Normal</td>
<td>ND</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Platelet membrane glycoprotein I</td>
<td>Normal</td>
<td>Abnormal</td>
<td>Abnormal</td>
<td>ND</td>
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</table>

ND, not determined or not described; RIPA, ristocetin-induced platelet aggregation in platelet-rich plasma from the patients; vWF, von Willebrand factor.

*The term “increased” means that smaller quantities of ristocetin induce platelet aggregation than in normal subjects.
†The term “increased” means a decrease in the threshold concentration of ristocetin required to induce the binding of normal vWF to patients’ platelets.
‡The term “increased” means an increased affinity of patients’ platelets for normal vWF.

patients. In addition, this report clearly demonstrated that there was considerable difference in the multimeric composition of plasma vWF among the patients: some showed a decrease in large multimers and others showed a decrease in both large and intermediate multimers (Figs 9 and 10).

Spontaneous platelet aggregation in vitro was observed in two patients from family B, which had never been described in this disorder before. The underlying mechanism of spontaneous platelet aggregation was not clear, but these patients had a greater amount of the intermediate vWF multimers in plasma (when plasmas of the same dilution were analyzed in parallel) and had higher plasma VIIIIR:RCO levels when compared with patients from family A. An explanation would be that their platelets probably bind the intermediate vWF multimers in their own plasma during stirring in the aggregometer cuvette, resulting in platelet aggregation.

The multimeric structure of vWF in patient platelets was normal. Moreover, the larger multimers appeared in the circulation after DDAVP infusion. The newly released vWF contained in plasma obtained after DDAVP infusion induced the aggregation of platelets from platelet-type vWD patients but not from normal subjects. These findings suggest that in these patients, there would be no abnormality in endothelial cells and megakaryocytes that synthesize vWF. As in type IIB patients studied by Ruggeri et al, the disappearance of the newly released larger multimers from the circulation was more rapid than in normal subjects and type I vWD. In the patients described here, however, the relative diminution of large (and intermediate) vWF multimers in plasma is probably the result of rapid removal from circulating plasma by adsorption on abnormal platelets, rather than a consequence of abnormal vWF function.

Studies that evaluate ristocetin-induced binding of vWF to platelets are important in the detection of a platelet abnormality. A consistent finding among these patients was increased ristocetin-induced binding of normal vWF to patient platelets (a decrease in threshold concentration of ristocetin required to induce the binding of normal vWF to patients’ platelets); this finding is specific to this disorder and is never seen in type IIB patients. Binding of patient plasma vWF to normal platelets was within the normal limit or decreased, probably based on the multimeric composition of plasma vWF.

Thus, four families studied by us, by Weiss et al, and by Miller et al can be entered into the same category. The heightened interaction between platelets and vWF, causing in vivo platelet aggregation, would be responsible for the lack of large multimer vWF in plasma and for the thrombocytopenia observed in these patients. The variations in platelet count and spontaneous platelet aggregation among the patients with platelet-type vWD may be attributable to the output of vWF from the endothelial cells and the multimeric composition of vWF remaining in circulating plasma after preferential binding of larger multimers to their abnormal platelets. The patients studied by Granlinc
et al.\(^{32,33}\) showed thrombocytopenia with large platelets and an increased affinity of patient platelets for normal vWF. However, their patients did not show platelet aggregation in response to normal vWF, suggesting that they would belong to a disorder different from platelet-type vWD studied by us and by others.\(^{9,10}\)

Considerable evidence implicates GPIb as a receptor for vWF.\(^ {34-37}\) Previous studies of this protein in platelet-type vWD have not demonstrated an abnormality.\(^ {9,10}\) Our study showed two separated bands in the GPI region, suggesting that GPIb is abnormal in the patients studied here. The calculated molecular weights of these abnormal bands were 150,000 and 140,000 and were different from that of abnormal glycoprotein observed in thrombasthenic platelets by Watanabe et al.\(^ {38}\) Electrophoretic abnormalities of GPIb have also been reported by Bolin et al.\(^ {39}\) and by Nurden et al.\(^ {40}\) These authors have shown that the GPIb in Tn syndrome had a faster migration than normal. The paper by Bolin et al.\(^ {39}\) is the first description of a doublet in the GPI region. However, the double band (denoted IA and IB) in their patients differs from that described here in that the first band corresponded in electrophoretic mobility to normal GPIb and the second had a faster mobility. The platelet receptor for vWF would be exposed on unstimulated platelets in platelet-type vWD.\(^ {23}\) We suggest that the platelet receptor abnormality in these patients is related to this abnormality of GPIb. Further study is required to clarify the relationship between these membrane abnormalities and the abnormal platelet reactivity.

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Further characterization of platelet-type von Willebrand's disease in Japan

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